



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 5/00, 5/08, C12P 1/00 A61F 2/00, C03B 19/00, 19/06 C03B 19/08	A1	(11) International Publication Number: WO 94/04657 (43) International Publication Date: 3 March 1994 (03.03.94)
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(54) Title: BIOACTIVE MATERIAL TEMPLATE FOR <i>IN VITRO</i> SYNTHESIS OF BONE TISSUE (57) Abstract Novel non-crystalline, porous bioactive glass and ceramic materials that permit the <i>in vitro</i> formation of bone tissue when exposed to a tissue culture medium and inoculated with cells are disclosed. The present invention also discloses methods of treating bioactive glass materials to control pH so that when the glass is exposed to a tissue culture medium and then inoculated with cells, bone tissue growth occurs <i>in vitro</i> . The glass material disclosed is preferably formed from SiO ₂ , CaO, Na ₂ O and P ₂ O ₅ and the porous, non-crystalline structure is most preferably created by melting the constituents, cooling and pulverizing the resulting glass, and then forming and hot pressing the powder. The glass of the present invention may be formed to produce templates that are useful for various indications, as well as granules that may be formed into a paste.		

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BIOACTIVE MATERIAL TEMPLATE FOR IN VITRO SYNTHESIS OF BONE TISSUE

The present invention relates to the synthesis of bioactive ceramic templates for optimum *in vitro* formation of bone and bone-like tissue. The fundamental mechanisms by which material surfaces elicit the *in vitro* response from bone cells are optimized, thereby leading to high formation rates of extracellular material with typical characteristics of bone tissue.

10 BACKGROUND OF THE INVENTION

Materials were first used to provide structural support during healing of bones, or to replace damaged or diseased bone tissue. Historically, the most important material selection criterion was inertness. It was believed that the implant material should only provoke the slightest reactions in the body. It is important to realize, though, that no matter how chemically inert a material may be, it always provokes a reaction upon implantation. The intensity of the reaction depends not only on the surface and bulk properties of the implant material, but also on the trauma at the time of surgery, the site of implantation, and the relative motion at the tissue-implant interface. This observation has prompted the use of "bioactive" materials instead of so-called inert materials. The implication is that a bioactive material must provoke a beneficial tissue response, specifically it must elicit the formation of the normal tissue at its surface and create an interface that promotes long functional life. Whereas the field of calcified tissue reconstruction has

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achieved this goal, this advance is not an end state, but merely a stepping stone for an even more ambitious goal: the creation of materials that are capable of serving as templates for *in vitro* bone tissue formation. This is part of the true
5 future of biomaterials: creating materials that, once inserted into the body, regenerate tissues rather than replace them.

Cell culture studies with osteoprogenitor cells or cells of osteoblastic phenotype have been performed, but never achieved acceptable results. Some of these prior studies did
10 not seek the optimization *per se* of extracellular material synthesis. Some prior studies used osteoprogenitor cells present in bone marrow extracts. Regardless of whether focus is placed on the determination of osteoblastic phenotype expression or elsewhere, these results can be used to determine
15 whether one of the resultant phenomena of osteoblastic phenotypic activity was extensive or not.

It is known to obtain bone marrow cells from the femora of young adult male Wistar rats by washing them out with α MEM (minimal essential medium) supplemented with 15% fetal
20 bovine serum, freshly prepared ascorbic acid, sodium β -glycerophosphate, dexamethasone (DEX) and antibiotics. See, Davies et al. "Early extracellular matrix synthesis by bone cells," Bone-Biomaterials Workshop, J.E. Davies Ed., University of Toronto Press, (December 1990). A quantity of this cell
25 suspension, e.g., 30 ml, containing cells from two femora, is aliquoted on to the material substrate. In a humidified 95% air - 5% CO₂ atmosphere the culture is maintained for a minimum of two weeks. It was shown that a calcified matrix of globular accretions, also containing sulphur, is formed. This layer was
30 typical for reversal lines in bone tissue, the cementum layer, and was considered by the authors as evidence that the calcified layer is the result of the expression of the osteoblastic phenotype by the cultured cells. Subsequently, there was what was called "frank bone formation." Thus, matrix
35 production can start within a time period of intermediate duration (17 days) by differentiating bone-derived cells *in vitro*. It has also been reported, however, that no calcified

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tissue formation has been obtained on porous ceramics. See Uchida et al. "Growth of bone marrow cells on porous ceramics *in vitro*," J. Biomed, Mat. Res. 21:1-10 (1987). The observation in the prior art with respect to the intrinsic
5 capability of cells to deposit a cement-like line is in any event certainly correct. The cell culture method described above is derived from Maniatopoulos et al.'s "Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats," Cell Tissue Res. 254:317-330 (1988), wherein this
10 particular cellular activity was shown to be present in cultures without any tissue stimulating biomaterial.

The effect of porous calcium phosphate ceramic on growth and hormonal response of periosteal fibroblasts, osteoblasts, and chondrocytes has been disclosed by other
15 workers. See, e.g., Cheng et al., "Growth of osteoblasts on porous calcium phosphate ceramic: an *in vitro* model for biocompatibility study," Biomaterials, 10, 63-67 (1989). As reported in this reference, the number of these cells increased 29-, 23- and 17-fold during a ten week time period.
20 Osteoblasts retained their phenotypic expression by producing only Type I collagens. Previously, Cheng had shown that the phenotypic expression of canine chondrocytes had been retained up to 13 months when cultured on porous hydroxyapatite ceramic granules. See Cheng, "In vitro cartilage formation on porous
25 hydroxyapatite ceramic granules," In Vitro Cellular & Developmental Biology, 21:6, 353-357 (1985). The elaboration of extracellular matrix reportedly started to appear at week one and increased throughout a thirteen month period.

Still others have studied the attachment and
30 subsequent growth of V79 cells in contact with various calcium phosphate ceramics and found that cell growth was markedly inhibited by hydroxyapatite, and slightly inhibited by tricalcium phosphate and glass ceramics. See Katsufumi et al., "The influence of calcium phosphate ceramics and glass ceramic
35 on cultured cells and their surrounding media," J. Biomed Mat. Res., 24:1049-1066 (1989). Under conditions of phagocytosis of small bioactive ceramic powders, RNA transcription and protein

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synthesis of osteoblast populations have been stimulated. See Gregoire et al. "The influence of calcium phosphate biomaterials on human bone cell activities: An *in vitro* approach," J. Biomed Mat. Res. 24:165-177 (1990). This phenomenon has also been observed for phagocytosing fibroblasts. It has been suggested that the increase of ³H-thymidine incorporation into DNA and the decrease of alkaline phosphatase activity probably resulted from secondary calcium messenger pathways. See Orly et al. "Effect of synthetic calcium phosphate on the ³H-thymidine incorporation and alkaline phosphatase activity of human fibroblasts in culture," J. Biomed Mat. Res. 23:1433-1440 (1989). Another study by Puleo et al., "Osteoblast responses to orthopaedic implant materials *in vitro*," J. Biomed Mat. Res. 25:711-723 (1991), provided inconclusive results regarding osteoblast attachment, osteoblast proliferation and collagen-synthesis. Another set of studies performed *in vivo*, documenting materials-dependent tissue response patterns are noteworthy. A series of experiments with porous hydroxyapatite and bone marrow cells was started by Ohgushi, Goldberg and Caplan and subsequently continued separately by Ohgushi and associates in Nara, Japan and Caplan and associates in Cleveland, Ohio (USA). See Ohgushi et al. "Heterotopic osteogenesis in porous ceramics induced by marrow cells," J. Ortho. Res., 7:568-578 (1989). These experiments demonstrate that the osteoprogenitor nature of the cells of a marrow cell suspension, implanted in heterotopic sites, are activated more readily when the suspension is infused into porous hydroxyapatite than when implanted by itself.

Finally, U.S. Patent No. 4,609,501 -- Caplan et al. discloses the stimulation of bone growth that includes the *in vitro* exposure of isogenic fibroblasts to a soluble bone protein capable of stimulating a chondrogenic response. The exposed cells are combined with a biodegradable carrier such as fibrin, although it is also suggested that the exposed cells may also be incubated with a prosthesis. A related Caplan et al. patent, U.S. Patent No. 4,609,551, discloses techniques for

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delivering the bone protein to anatomical sites, while U.S. Patent No. 4,608,189 also to Caplan et al. discloses processes for obtaining suitable bone protein.

The present invention is focused on substrate materials and shows that modification of the material used as the substrate can lead to major differences in amount and rate of tissue formation *in vitro*. It is thus an object of this invention to synthesize ceramic materials which serve as the ideal templates upon which life processes, specifically, bone tissue formation, can thrive.

SUMMARY OF THE INVENTION

These and other objects are met by the synthesis of porous glass without producing significant crystallization and the conditioning of the glass surface such that cell attachment is enhanced and extensive extracellular matrix (ECM) formation can take place. Control of the bioactivity reactions to produce pH variation in solution within boundaries that do not kill the cells once seeded on the porous glass is also disclosed.

Generally, the present invention discloses a bioactive material surface treated to enhance bone cell attachment and activity when the material is placed in a tissue culture medium, such that when inoculated with cells, bone tissue forms *in vitro*. Preferably, the bioactive comprises a glass treated to control pH, most preferably to less than 7.6. in a preferred embodiment, the bioactive material is a non-crystalline glass consisting essentially of: SiO_2 ; CaO ; Na_2O ; and P_2O_5 having the following preferred composition: 45% by weight SiO_2 ; 24.5% by weight CaO ; 24.5% by weight Na_2O ; and 6% by weight P_2O_5 . Alternatively, in other preferred embodiments, the bioactive material of the present invention comprises a ceramic. In either glass or ceramic forms, however, the material is preferably porous, and the porosity is between about 20-30% and the pore size range is about 75-200 μm .

The present invention therefore discloses methods of forming a porous glass substrate comprising the steps of

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melting an admixture consisting essentially of SiO_2 , Na_2O , CaO and P_2O_5 and then quenching the melted admixture to create a glass frit. A glass powder is formed from the glass frit that preferably has a particle size between about 40 to 70 μm . A
5 porous glass substrate is then formed from this powder by a foaming process. An important aspect of the present invention is that the resulting porous glass exhibits substantially no crystallization. Crystallization can occasionally be acceptable, but its main drawbacks are the nonuniform corrosion
10 rates of the surface, and therefore the spatial variation of the reaction layers. Furthermore, the corrosion reactions are considerably slower, thereby necessitating long conditioning and incubation times. Alternatively, the porous glass substrate can be formed by creating a slurry of glass powder
15 and a binder such as polyvinyl alcohol and pouring the slurry into a mold, drying the slurry and sintering it to produce a porous glass substrate. Finally, the porous glass substrate can be formed by first quenching the admixture so that a glass is formed. This glass is transformed into a glass ceramic
20 having more than one crystalline phase and then preferentially dissolved to result in a porous material. The preferential dissolution may be accomplished by, for example, adding a solvent such as an acid or a base.

The present invention thus discloses the formation of
25 a porous, preferably non-crystalline, bioactive glass. It is anticipated, though, that any known bioactive glass or ceramic can suitably be transformed to become a template for *in vitro* synthesis of bone tissue. Inoculation times will probably differ substantially. The glass material disclosed herein can
30 be formed into a prosthetic article such as a disk, a sleeve, a rod or any other form of substrate or template. Alternatively, the glass formed herein can be provided in the form of particles.

In order to enhance bioactivity and bone formation
35 using the glass of the present invention, the glass must be treated to prepare its surface for cell attachment and to control pH prior to its inoculation with cells. Therefore, the

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present invention also discloses methods of forming bone tissue comprising the steps of providing a porous bioactive template consisting of the glass material described above, immersing the template in a buffer solution and immersing the template in a tissue culture medium to produce a surface which significantly enhances bone cell activity when cells are inoculated on this surface. Finally the template is inoculated with cells and bone tissue is permitted to form thereon. Most preferably, the buffer solution is buffered at a pH of about 6.8 and the tissue culture medium has a pH not exceeding about 7.6. The cells inoculated on the template may be chosen from any preparation providing osteoprogenitor cells, bone marrow cell preparations, cells of osteoblastic phenotypic potential or of osteoblastic phenotype. Inoculation may also proceed with fibroblasts and chondrocytes.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a photomicrograph of a cross-section of a substrate made in accordance with the present invention illustrating the formation of bone-like sheets.

FIG. 2 is a photomicrograph of the surface of bioactive glass made in accordance with the present invention showing globular accretions connected by collagen fibrils.

DETAILED DESCRIPTION OF THE INVENTION

In the field of *in vivo* bone growth, glasses are known to produce a beneficial effect beyond what calcium phosphate ceramics produce. See e.g. Schepers et al. "Bioactive glass particulate material as a filler for bone lesions," J. Oral Rehabilitation, 8, 435-452 (1991). However, this effect *in vivo* has not been reproduced *in vitro*. The present invention discloses a new physical form of glass along with an additional new surface treatment that is effective *in vitro*.

Although porosity of a substrate is not essential, it is advantageous in view of the large specific surface area in potential contact with cells. The growth of tissue through the substrate leads to a fast formation of tissue needed for

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reimplantation and the artificial material degrades faster. One aspect of the present invention therefore provides methods for making bioactive glass porous. It will be realized that the methods as proposed in the prior art would have produced a crystallized glass, and crystallization must be avoided since it interferes with bioactivity reactions. In other words, the dissolution and ion exchange reactions leading to a calcium-phosphate surface, possibly a carbonated calcium-deficient hydroxyapatite very similar to the mineral phase of bone, are slowed down substantially with crystals being present in the glass. In the limit, it is possible that the necessary calcium-phosphorus rich layer would be formed so slowly that it is of minimal or no consequence to the cellular attachment reaction. If cellular attachment is not achieved, it can be assumed that cellular function will be hampered, if not impeded. Thus, when specifically focusing on the secretory function, that is the extracellular matrix formation or the bone tissue-like synthesis, it would be severely reduced and, therefore, no true utility would be achieved. It should be noted at the outset that the present invention does not rely on cell biology. Many cells that have the potential to be upregulated to cells of the osteoblastic phenotype can be used.

A. GLASS PREPARATION

Bioactive glass with a nominal composition (% by weight) 45% SiO₂, 24.5% Na₂O, 24.5% CaO and 6% P₂O₅ represents a most preferred embodiment and is prepared from chemically pure reagent grade Na₂CO₃, CaCO₃, Ca₁₀(OH)₂(PO₄)₆ and SiO₂. However, the following ranges of these constituents are useful to form the material of the present invention: 40-60% SiO₂; 5-30% Na₂O; 10-35% CaO and 0-12% P₂O₅. The prorated amounts of the reagents are admixed and melted in a crucible at about 1350 C. The homogeneity of the melt is assured by swirling the melt-containing crucible as required, but most preferably at least three times. The melt is then poured into deionized water. The glass frit obtained by this method is then dried and used for the synthesis of porous glass. For the synthesis

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of porous glass, the glass frit is crushed and ground into a powder with a particle size in the range of about 40-70 μm . This powder is admixed with 2-3% CaCO_3 , which is used as foaming agent. The mixture is hot pressed in vacuum at about 5 50 MPa and 460 C for about two hours. The resulting pore size range is about 70-200 μm . A larger pore size range, e.g., about 200-500 μm , is obtained by using either CaCO_3 powder of a larger particle size, or by using NaHCO_3 as the foaming agent. The average porosity and pore size is determined by 10 weight measurements and stereometry, respectively. An X-ray diffraction analysis of porous bioglass prepared in accordance with the present invention shows complete vitrification with no sign of crystallization after the foaming process.

* * *

15 In order to optimize the synthesis of glass in accordance with the present invention, several foaming agents (Na_2CO_3 , NaHCO_3 , $\text{NH}_4\text{H}_2\text{PO}_4$ and marble) with different concentrations (1%, 2%, 3%, 6% and 10%) and a number of sintering and foaming treatments (at 450, 580, 475 and 550 C) 20 for different periods of time (1, 2, 3, and 5 hours) were used. Double stage heat treatment (450 C for 1 hr; then heated at either 475 C, 550 C, or 580 C for 1 hr) was also used. Glass powder of different grain sizes, e.g., about 110 μm and between about 70 - 40 μm were used and the applied pressure was also 25 taken as a variable and pressures of about 100, 70, and 40 MPa were tried. Those foaming agents and processing conditions that did not measurably initiate a glass crystallization reaction are preferred. Any crystallization renders glass more corrosion resistant or produces a nonuniform corrosion. This 30 must be avoided as it adversely affects the beneficial formation of the surface reaction layers.

The best result was obtained by admixing the glass powder of grain size between about 40 and 70 μm with about 2.3% CaCO_3 that is pressed in a graphite die at a pressure of about 35 50 MPa. The pressed admixture is then heated at about 460 C for about two hours in a vacuum press. Using high pressure, e.g., about 100 MPa and heat treatment at about 580 C produces non-

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porous glass-ceramic material, so these values for these variables are not preferred. Moreover, it was found that using low pressure, e.g., about 40 MPa, produces a fragile material that can not be handled. Heat treatment of long duration will
5 initiate crystallization of the glass, especially in the presence of a foaming agent, so the shortest heat treatment duration represents a most preferred embodiment.

Alternative processes useful with the present invention to produce porous glass, glass-ceramic, or ceramic
10 are:

(1) Foaming and Sintering

By making an aqueous ceramic slurry with a binder (e.g., polyvinyl alcohol), and then mixing the slurry with a foaming agent (e.g., H_2O_2 or $CaCO_3$) pouring the mixture into a
15 mold, allowing it to dry, and then sintering it at high temperature useful material may be produced. This method is less preferred because it can change the nature of the glass surface and hence limit the bioactivity.

(2) Ceramization and Leaching

20 By crystallizing the glass into more than one crystalline phase, and then treating the obtained glass ceramic with acid or base that preferentially dissolves one phase and leaves the others, porous material is created. The ceramization and leaching process represents a less preferred
25 embodiment, however, because leaching of calcium phosphate-rich phase will decrease the bioactivity of the glass.

(3) Sol Gel Method

As well known in the art, controlling the heat treatment cycle of the ceramic or glass gel controls the pores and interpores of the material and thus a porous ceramic can be
30 obtained. However, the control of pore size and porosity percent of bioactive glass prepared by this method is much more difficult to obtain in the desirable range than sintering and foaming process described above.

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B. CONDITIONING OF THE GLASS SURFACE AND CONTROL OF pH VARIATION

Another important aspect of the present invention is the conditioning of the glass surface with concurrent pH control of the solution. Such conditioning is essential for two reasons: (1) if cells are added to unconditioned glass, the glass corrosion reaction which takes place would lead to pH values at the surface of the glass and in the bulk of the solution that would kill the cells; and (2) the treatment must make the surface ready for expedient cell attachment and extracellular matrix deposition. Before describing the best conditioning of the glass we first describe in a chronological fashion, some of the experiments performed.

Glass disks made in accordance with the techniques described above were soaked in tris buffer solution containing ions in a concentration similar to that found in interstitial fluid, and shaken on a rotary table at 37 C (pH 6.84) for 48 hours. The volume of this solution was one liter. No change in the pH value was measured during the time period of glass immersion. Electron dispersive X-ray analysis (EDXA) revealed that a calcium phosphate rich layer had formed on the surface of the bioactive glass.

In another experiment, the glass disks were either treated in modified tris buffer solution for 20 hours, or treated for 20 hours in tris buffer followed by 48 hours in a typical tissue culture medium (TCM), i.e. E-MEM (Eagles' minimal essential medium + 10% NBS + 2 mM L- glutamine) (treatment TB2-E-MEM), then inoculated with cells. Four or five disks were immersed. The solution was maintained at 37 C. A pronounced difference was observed in number of cells attached to the glass disks and secretion of extracellular matrix material. Comparison of the number of cells attached to these two differently treated glasses showed more cells adhered to the glass disks treated in tris buffer alone. Both sets of samples showed collagen fibrils and calcified nodules, yet, neither showed any bone-like tissue formation. The cells attached to the glass disks treated according to TB2-E-MEM produced more collagen fibrils in between the cells. Since

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cell attachment represents a preferred embodiment, in our subsequent experiments we now assumed that immersion in the modified tris solution was preferable. In this next set of experiments we then addressed the question whether, if we
5 inoculated cells on these disks after treatment in modified tris for 20 hours, there would be a pH variation with the potential of killing of the cells. In these experiments we treated the disks for 20 hours in modified tris and subsequently immersed the disks in the same tissue culture
10 medium as we would use for the inoculation and incubation of cells and we measured the pH variation. We did this first for one ratio of the tissue culture medium volume to glass weight. Thus, we treated glass disks in tris buffer at 37 C for 20 hours followed by immersion in a E-MEM at 37 C. The ratio of
15 solution volume to glass weight was about 19 ml/g. The corrosion products of the glass were found to increase the pH of the TCM solution from 7.6 to 7.9 after 24 hours, and from 7.6 to 8.0 after 48 hours. This increase in pH may have an adverse effect on cells if they were inoculated in such
20 solution, i.e., the cells would die.

A next series of experiments used different ratios of TCM volume to glass weight. Based on the results of the last experiment, we also increased the duration of immersion in tris from 20 to 48 hours. This was intended to minimize subsequent
25 dissolution from the glass into the TCM, when cells would be present. As before, there was a two step immersion: immersion in modified tris buffer solution for 48 hours, and then immersion in TCM for three days. The initial pH of the TCM is 7.6. The pH of the TCM was measured after 24, 48, and 72
30 hours. The TCM was then replaced by TCM + 3 mM B-glycerol phosphate, and the pH was also measured every 24 hours for 3 days. The minimum TCM volume/glass weight ratio that did not change the pH value of the TCM was 90 ml/g, yet there may still be a pH gradient between the glass surface and the bulk of the
35 TCM. Furthermore, the pH value of the TCM inside the pores may be considerably higher than the one of the TCM in the bulk of the solution in the dish. We found that when the pH of the

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bulk TCM raised to about 7.85, for example, due to a low ratio of TCM volume to glass weight, the activity of the cells that were seeded on the preconditioned glass disks was greatly inhibited. Scanning electron microscope (SEM) analysis of
5 these cultures showed very limited cell processes and the alkaline phosphatase activity was minimal, as followed from the faint pink color seen when samples were stained with fast red staining.

Based on the these last results, we performed yet
10 another experiment in which we now added a strong biological buffer to the tissue culture medium: we added 20 mM Hepes. We added this buffer in order to stabilize the pH value inside the pores. Thus, the total set of conditions was: immersion for 48 hours in modified tris, immersion in E-MEM complemented with
15 20 mM Hepes and a 90 ml/g ratio of TCM volume to glass weight. The second immersion, then, represents conditions for inoculation. Thus, when the pH in the pores and at the surface of the glass was stabilized at 7.6 during the period the cells were seeded on the glass, abundant cell processes were possible
20 and bone like tissue formed extensively after only one week in culture.

The glass disks are dried, and most preferably sterilized by ethylene oxide prior to inoculation with cells.

The effect of the immersion treatment being the
25 formation of calcium phosphate rich reaction layer is known. See Hench et al. "Bonding mechanisms at the interface of ceramic prosthetic materials," J. Biomed. Mater. Res. Symp., 2:117-141 (1972). This reaction layer gradually matures into a poorly crystallized, defective calcium hydroxyapatite
30 structure. See Kim et al. "Early stages of calcium phosphate layer formation in bioglass," J. Non-Cryst. Solids, 113:195-202 (1989). The immersion treatment of the present invention, however, differs in a fundamental way from the method used by Hench et al., referenced above. The intent of the prior art
35 was to establish kinetics of the several ion exchange, dissolution, condensation, precipitation and transformation reactions at the glass surface. For these reasons, a tris-

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buffered solution which does not contain any of the typical components of interstitial bone fluid was used. In contradistinction, the present invention achieves a glass surface resembling the mineral phase of bone upon which biomolecules are absorbed as soon as the glass is brought into contact with a solution containing biological molecules. This can be achieved by either of two ways: i) prior to inoculation but after immersion in modified tris buffer, the disks are immersed in TCM or any other suitable solution containing biological molecules normally occurring in bone tissue; or ii) the disks are immersed in the cell suspension containing biological molecules. Before the cells adhere, the biological molecules attach to the glass surface and incorporate into the continuously developing glass surface reaction layer. The result is that the developing calcium phosphate layer is coated and intimately mixed with organic molecules.

C. CHARACTERIZATION OF IN VITRO SYNTHESIZED BONE TISSUE

After the above described modified tris buffer pretreatment, the 10 mm diameter disks are placed in 60 mm diameter petri dishes or other suitable container, wetted with TCM and inoculated with about one million neonatal rat calvaria osteoblasts from a suspension containing 1.2 million cells/ml. The cell suspension used herein was obtained by an enzymatic digestion method using 0.2% collagenase. To allow for cell attachment, the petri dishes were kept in the incubator for about one hour prior to flooding them with TCM. Two days later, the TCM was exchanged by TCM supplemented with 3 mM β -Glycerol phosphate. This exchange was repeated at day four. The dishes were sacrificed at day seven. Morphological analysis by using scanning electron microscopy (SEM), energy dispersive x-ray microanalysis (EDXA) for calcium, phosphorus and sulfur, qualitative and quantitative determination of alkaline phosphatase concentration in solution, and DNA, collagen I and osteocalcin synthesis reveal the properties of the tissue that had formed.

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The porous specimens were found to be totally invaded by the cells and the extracellular matrix they elaborated, as seen in FIG. 1. FIG. 1 shows a scanning electron micrograph (x100) of a cross-section through the porous bioactive glass after 1 week of incubation. Bone-like sheets were formed throughout the whole thickness of the porous glass sample. FIG. 2 shows another scanning electron micrograph (x3500) of the bioactive glass surface. The photograph was made at a time the parameters of our invention were not yet optimized. It shows some of the events prior to the extensive bone tissue formation: globular accretions in direct contact with the glass surface are visible. Collagen fibrils interdigitate with these globular nodules. The calcified cement-like layer is not yet covered by the bone-like sheets and is thus still visible.

The qualitative assessment of alkaline phosphatase activity reveals that the highest activity is with the cells on the bioactive glass disks; there is only a faint activity on the bottom of the petri dishes around the glass disk.

The quantitative determination of the alkaline phosphatase activity expresses an average per dish. Using the method of quantifying released para-nitrophenol (PNP) set forth in Lowry et al. "The quantitative histochemistry of the brain: II. Enzyme measurements," J. Biol Chem., 207:19-37 (1954), a rate of 0.62 nmol pNP/min/ μ g protein was found. As for μ g DNA, a typical value (2.01 μ g/ml) for osteoblastic phenotypic expression was indicated. The extensive morphologic observation of the various samples suggests the following hypothetical sequence of events: in a first step the foci of calcification form: small globules, 1-2 μ m in diameter, appear on the glass surface. Subsequently, collagen fibrils are produced and attach to the calcified nodules.

It should be noted that collagen I synthesis has been confirmed. From thereon, the synthesis of both calcified substance and intertwined fibrils continues, thereby leading to the gradual formation of bundles and eventually the coalescence into sheets of bone-like material. It is important to note that the surface of these sheets is smooth and free of

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calcified globules. Such observations are intriguing as they fit the theory that these calcified globules serve to bridge the collagen fibrils and thereby create the continuous bone-like sheets. It is another intriguing observation that without
5 the initial elaboration of these nodules on the glass surface, the cells do not produce collagen fibrils. Osteocalcin radioimmunoassay (using goat anti-rat osteocalcin, donkey anti-goat second antibody and I-125 rat osteocalcin) showed a considerable concentration (about 8 ng/ml) of osteocalcin.
10 Osteocalcin was detected only on those dishes containing glass and osteoblasts. Control dishes with osteoblasts but without glass disks never showed appreciable concentration of osteocalcin.

The calcification of elaborated material was not the
15 result of a physio-chemical phenomenon of precipitation from a solution supersaturated in calcium and phosphorus, this in itself resulting from glass dissolution. Control experiments with glass without inoculation of cells, or cell culture experiments without porous bioactive glass did not produce a
20 measurable calcification.

The applications of the *in vitro* synthesis templates disclosed herein may be described by analyzing the advantages and disadvantages of granules of bioactive glass. See Schepers et al. "Bioactive glass particulate material as a filler for
25 bone lesions," J. Oral Rehabilitation, 8, 435-452 (1991). One of the great advantages of using the glass in the form of granules is that they are presently ready for clinical use, and the *in vitro* synthesis templates are not. Since both are glass based products, the acceptance in the market of granules may
30 greatly help the future introduction of the templates.

It is worthwhile to note that the template disclosed herein can be made in porous particle form. This enables the templates to be used for filling the many different forms of defects one encounters in surgery. However doing so forgoes
35 one of the significant attributes of the templates, namely that they can come as large rigid structures. This is of great significance in any situation of either trauma or pathology,

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where bone loss is substantial and where the repair and reconstruction must be attempted with solid blocks to give the necessary rigidity to the structure after surgery. In such situations it is a limitation of granules or particles that
5 they form a paste, since they can be squeezed from the site of delivery.

A second major advantage of *in vitro* synthesized bone tissue formation on templates made in accordance with the present invention is that they can be implanted into sites with
10 otherwise limited growth potential. Cells which have been seeded onto the template in accordance with the methods of the present invention have been made active before implantation. This observation opens up enormous perspectives. Our Western populations are growing ever older and it is in this older age
15 group that osteoporosis is prevalent. Osteoporosis, characterized by greater bone resorption than formation, afflicts more than fifty percent of the population beyond fifty-five years of age. It leads to the gradual compression of the spine and the typical hunch back. Osteoporosis is also
20 the basis of a large percentage of hip fractures, with their considerable morbidity, and even significant secondary mortality. In short, the seeded glass surfaces with *in vitro* grown bone tissue disclosed herein can be inserted in all areas of the body that exhibit an increased risk of fracture and a
25 decreased or even extinct potential for bone tissue formation. The network of porous, bioactive glass of the present invention can be made such that it resembles the porous bone network in the vertebrae of the spine, or the neck of the hip bone.

The cells seeded on to the synthetic substrate
30 disclosed herein are most preferably cells from the patient. In experiments, osteoblasts, i.e., the cell which secretes the bone tissue material, have been used. However, as well known to those of ordinary skill osteoblasts are merely the end stage cell of cells of osteoblastic lineage. It is also possible, to
35 use cells which are precursor cells to osteoblasts, that are typically present in the bone marrow. A simple extraction, followed by an appropriate separation and conditioning, yields

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cells that can be seeded. Intra-family donation of these cells are planned for those cases where either the aging of the patient to be treated has progressed to the point where he has only a limited cell activity anymore, or where the clinical
5 circumstances are such that cell biopsies cannot be obtained.

A third important advantage of the porous bioactive glass templates disclosed herein is that they can be implanted in sites where there is an immediate need for bone. Once the templates have been seeded, results indicate that bone tissue
10 has already formed extensively *in vitro* after one week.

In summary, the present templates will not fully replace the nonporous granules discussed in the Schepers reference noted above, rather they will broaden the indications for which these granules were used before in a rather
15 restrictive way. Furthermore, they will create totally new therapies, such as the ones in osteoporotic pathology as suggested above.

Those of skill in the art will recognize that these porous templates will also become used in primary joint
20 replacements. A porous coated prosthesis will be surrounded by a sleeve of the porous template material made in accordance with the present invention, and seeded with cells. After about one to two weeks, it will be inserted into the patient. At that time, a very expeditious incorporation of the device into
25 the native femoral bone will occur.

Another new and useful area to which the present invention can be applied is the delivery of drugs or biological molecules to a patient. By adding a drug or biological molecules to the substrate, other therapeutic effects in
30 addition to bone replacement can be achieved.

Although certain embodiments and applications of the present invention have been set forth with particularity, review of the foregoing specification will lead those of ordinary skill to realize that the present invention is not so
35 limited. Numerous variations, modifications and application to other indications will immediately become apparent that do not depart from the spirit of the invention. For this reason,

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reference should be made to the appended claims in order to ascertain the true scope of the present invention.

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What is claimed is:

1. A bioactive material for the *in vitro* inoculation of cells capable of attaining an osteoblastic phenotype comprising a substrate having a surface, characterized by said
5 surface having been treated prior to inoculation under aqueous conditions effective to render the substrate incapable of raising the pH of a tissue culture medium above a physiological pH upon inoculation.
2. The bioactive material of claim 1, wherein the
10 material comprises a glass.
3. The bioactive material of claim 2, wherein the glass comprises a non-crystalline glass consisting essentially of: SiO_2 ; CaO ; Na_2O ; and P_2O_5 .
4. The bioactive material of claim 3 having the
15 following composition: 40-50% by weight SiO_2 ; 20-30% by weight CaO ; 5-30% by weight Na_2O ; and 0-12% by weight P_2O_5 .
5. The bioactive material of claim 1, wherein the material is porous and exhibits a porosity between about 10-80%, and the pore size is less than 800 μm .
- 20 6. An implantable bone replacement comprising a substrate and bone tissue formed on the substrate *in vitro* by inoculation with cells capable of attaining an osteoblastic phenotype, characterized by said substrate having been treated prior to inoculation under aqueous conditions effective to
25 render the substrate incapable of raising the pH of a tissue culture medium above a physiological pH upon inoculation.
7. The implantable bone replacement of claim 6, wherein the substrate comprises a porous, bioactive ceramic.
8. The implantable bone replacement of claim 7,
30 wherein the substrate is in the form of a sheet.

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9. The implantable bone replacement of claim 7, wherein the substrate is in the form of granules.

10. A method of forming a porous glass substrate for use in an implantable bone replacement in accordance with any
5 of claims 6-9, comprising the steps of:

melting an admixture consisting essentially of SiO_2 , Na_2O , CaO , and P_2O_5 ;

quenching the melted admixture to create a glass
frit;

10 forming a glass powder from the glass frit; and

forming a porous glass substrate from the glass powder, whereby the resulting porous glass exhibits substantially no crystallization.

AMENDED CLAIMS

[received by the International Bureau on 13 December 1993 (13.12.93);
original claims 1 and 6 amended; other claims unchanged (1 page)]

1. A bioactive material for the *in vitro* inoculation of cells capable of attaining an osteoblastic phenotype comprising a substrate having a surface capable of leaching ions into a tissue culture medium wherein said surface is treated prior to inoculation under aqueous conditions effective to render the substrate incapable of raising the pH of a tissue culture medium above a physiological pH upon inoculation.

2. The bioactive material of claim 1, wherein the material comprises a glass.

3. The bioactive material of claim 2, wherein the glass comprises a non-crystalline glass consisting essentially of: SiO_2 ; CaO ; Na_2O ; and P_2O_5 .

4. The bioactive material of claim 3 having the following composition: 40-50% by weight SiO_2 ; 20-30% by weight CaO ; 5-30% by weight Na_2O ; and 0-12% by weight P_2O_5 .

5. The bioactive material of claim 1, wherein the material is porous and exhibits a porosity between about 10-80%, and the pore size is less than 800 μm .

6. An implantable bone replacement comprising a substrate and bone tissue formed on the substrate *in vitro* by inoculation with cells capable of attaining an osteoblastic phenotype wherein said substrate has a surface capable of leaching ions into a tissue culture medium and said surface is treated prior to inoculation under aqueous conditions effective to render the substrate incapable of raising the pH of a tissue culture medium above a physiological pH upon inoculation.

7. The implantable bone replacement of claim 6, wherein the substrate comprises a porous, bioactive ceramic.

8. The implantable bone replacement of claim 7, wherein the substrate is in the form of a sheet.

STATEMENT UNDER ARTICLE 19

Claims 1 and 6 are amended. Both claims have been amended to reflect that the substrate have a surface capable of leaching ions into a tissue culture medium. These amendments were made for purposes of clarification. Support can be found in the discussions of bioactivity reactions at page 5, lines 12-19; page 8, lines 3-11; and page 11, lines 3-9 of the international application as filed. Accordingly, the amendments do not go beyond the disclosure of the international application as filed.

1/1

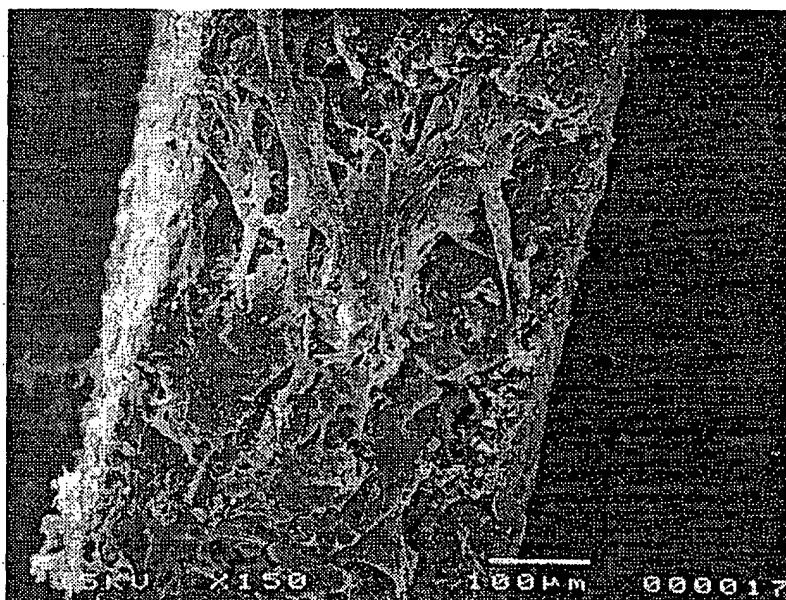


FIG. 1

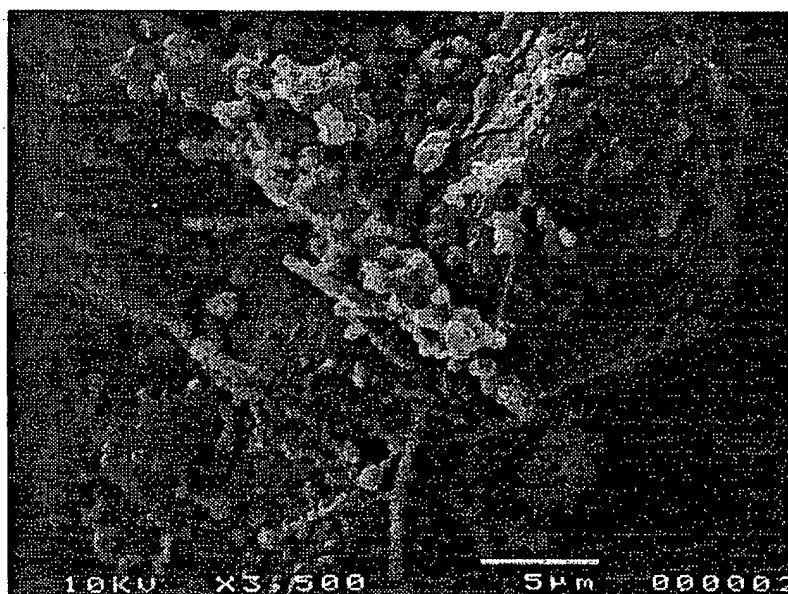


FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07583

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12N 5/00, 5/08; C12P 1/00; A61F 2/00; C03B 19/00, 19/06, 19/08

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.1, 240.2, 240.21, 41; 424/422; 65/18.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Biosis, Medline, Chem Ab, Derwent WPI, Embase, Pascal, search terms: bone, glass, ceramic, pH, in vitro, osteoblast, osteogenic, inventor names, bioactive material, tissue culture media

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Acta Orthopedics Scandinavia, Volume 61, Number 5, Issued May 1990, H. Ohgushi et al., "Osteogenic capacity of rat and human marrow cells in porous ceramics", pages 431-434, see entire document.	1-10
Y	Biomaterials, Volume 13, Number 6, Issued June 1992, W.C.A. Vrouwenvelder et al., "Behaviour of fetal rat osteoblasts cultured in vitro on bioactive glass and nonreactive glasses", pages 382-392, see entire document.	1-10
Y	Biomaterials, Volume 8, Issued July 1987, T. Matsuda et al., "The in vitro response of osteoblasts to bioactive glass", pages 275-284, see entire document.	1-10

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
20 September 1993

Date of mailing of the international search report
12 OCT 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07583

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category ^a	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Biomedical Materials Research, Volume 24, Issued April 1990, T. Kokubo et al., "Solutions able to reproduce in vivo surface-structure changes in bioactive glass-ceramic A-W", pages 721-734, see entire document.	1-10
Y	R.I. Freshney, "ANIMAL CELL CULTURE, A PRACTICAL APPROACH", published 1986 by IRL Press (Oxford), pages 39-41, see entire document.	1-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07583

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/240.1, 240.2, 240.21, 41; 424/422; 65/18.3

Laurie Hall

To: Maryanne Fenerjian

Subject: RE: Case No. 1438 - entity status

From: Maryanne Fenerjian [mailto:maryanne_fenerjian@hms.harvard.edu]

Sent: Wednesday, October 30, 2002 4:00 PM

To: John Iwanicki

Cc: Kellie.Watson@pyrosequencing.com; PerJohan.Ulfendahl@pyrosequencing.com; Laurie Hall; janet_maher@hms.harvard.edu

Subject: Case No. 1438 - entity status

Dear John,

Recently, we discussed the rate at which Harvard should be paying official fees in Case No. 1438 ("Replica Amplification of Nucleic Acid Arrays") in light of the license to PyroSequencing.

Although the entire case is listed under the "Patent Rights" definition in the license, only some of the applications contain claims that are within PyroSequencing's licensed field of use. The remainder were listed because they could serve as the basis for future applications that do contain claims within the field. Under the license, Harvard will receive reimbursement from Pyrosequencing only for costs incurred in association with applications that contain claims that the company is licensed to practice. You have confirmed that patents and applications that contain no claims that PyroSequencing has the right to practice are entitled to small entity status.

So that we pay fees at the proper rate for all patents and applications, the breakdown of is as follows:

Containing claims within PyroSequencing's licensed field:

PCT/US00/06390 (WO 00/053812 A3), your reference 10498/87039

Australian Patent Application Serial No. 38761/00, your ref. 10498/00023

Canadian Patent Application Serial No. 2,370,535, your ref. 10498/00024

European Patent Application Serial No. 00917853.4, your ref. 10498/00025

Japanese Patent Application Serial No. 2000-603433, your ref. 10498/00026

U.S.S.N. 09/522,732 (allowed), your ref. 10498/85926, and

all divisional applications now being filed on these cases with claims to compositions.

Not containing pending or issued claims within PyroSequencing's licensed field:

10/30/2002

U.S.S.N. 60/061,511 (abandoned in favor of utility before execution of license; costs reimbursed by Mosaic, a small entity)

U.S.S.N. 60/076,570, (same)

U.S.S.N. 09/143,014 (issued as U.S. Patent No. 6,432,360), your ref. 10498/75641

U.S.S.N. 09/267,496 (allowed), your ref. 10498/78230

PCT/US98/17862 (WO 99/19341, abandoned prior to execution of license; corresponding national phase filings to be abandoned through inaction as deadlines come due), your ref. 10498/75640

By copy of this e-mail, I am asking Kellie Watson and PerJohan Ulfendahl to review my assessment regarding the scope of claims in the patent and applications listed in this second category, and to let me know whether they believe that any of the claims fall into the licensed field (i.e., nucleic acid analyses using the dithiol-linked, cleavably-labeled nucleotides of the invention without the use of the polony technology).

As you also are aware, PyroSequencing held rights under a sublicense with Mosaic from mid-March of 2001 through May 31, 2001, at which time Harvard terminated Mosaic's license. Mosaic's last patent reimbursement to Harvard was for costs invoiced to them on January 2, 2001. Because PyroSequencing now holds a field-exclusive license to this invention, they will reimburse Harvard for costs within the field back to the point at which Mosaic stopped paying. Some of those costs predated the sublicense by a number of weeks.

We will need your opinion on whether official fees paid on actions taken between 12/28/00 (the date of the last Banner invoice for which Harvard received reimbursement from Mosaic) and the effective date of PyroSequencing's sublicense should be paid at the large entity rate, given the present reimbursement structure. I would assume that fees paid during the sublicense term should be brought up to the large entity rate. We also will need your opinion on whether fees paid between May 31, 2001 and the effective date of the present license (July 15, 2002) should be brought up to the large entity rate. The issue is that while Harvard and PyroSequencing never formally came to an agreement as to whether PyroSequencing's sublicense terminated with the underlying Mosaic license, and have no further need to do so, we must consider the question in terms of whether someone could challenge the validity of the patents based on an argument that the sublicense remained in effect until the new license was executed and that, as a result, large entity fees should have been paid. Please advise.

As always, thank you for your help.

Best regards,

Maryanne

10/30/2002